Optimal conditions for mycelial growth of *Schizosaccharomyces japonicus* cells **in liquid medium: it enables the molecular investigation of dimorphism**

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Abstract

The non-pathogenic dimorphic fission yeast, *Schizosaccharomyces japonicus* could be a suitable model organism for investigation of the genetic background of mycelial growth, as it has haploid chromosome set and its genome is sequenced. Since earlier results have suggested that its morphological transition required solid substrates, but molecular biological experiments would require hyphae production in a liquid medium, we wanted to find circumstances which enable hyphae production in liquid media.

Several external conditions were investigated, but the strongest inducer was Fetal Bovine Serum. Its positive effect could be hampered by heat and was dependent on pH, temperature and concentration of the serum. Other protein containing compounds such as peptone and BSA or amino acids proved to be ineffective or weak. Generally, the uninduced and induced mycelial growth of *Sch. japonicus* could be improved by lower external pH and higher temperature.

Introduction

An important feature of pathogenic yeasts is their ability to switch between different morphological forms. Their filamentous forms are thought to be critical for their pathogenesis (Molero *et al*., 1998; Whiteway and Oberholzer, 2004), thus the genetic and molecular analysis of morphological transition can be very important.

The morphological switch involves different processes, such as the MAP kinase cascade and the cAMP dependent pathway (Gancedo, 2001), a changeover to unipolar growth (Sipiczki *et al*., 1998a), reorganisation of cytoskeleton (Yokoyama *et al*., 1994), repression of cell separation (Balazs *et al.*, 2012), changes in the state of DNA (Furuya and Niki, 2010) or environmental sensing (reviewed in Ernst, 2000 and Biswas *et al*., 2007). Important environmental factors are for instance, nutrient limitation (Gimeno *et al*., 1992; Lopez-Bergez *et al*., 2010), a poor carbon source (Zaragoza and Gancedo, 2000), stress conditions (Dickinson, 1996; Hornby *et al.*, 2004), changing in pH values (Penalva and Arst, 2002), a sudden change in temperature (reviewed in Sudbery *et al*., 2004) or in oxygen concentration (Dumitru *et al.*, 2004).

Schizosaccharomyces japonicus is believed to be an attractive model organism for investigation of the genetic background of dimorphism, as it is non-pathogen, it has a haploid chromosome set and its genome is also sequenced (www.broadinstitute.org). Its further good features were recently summarised (Klar, 2013; Niki, 2014). This species belongs to the *Schizosaccharomyces* genus, and its ancestors separated about 420 to 330 million years ago from filamentous ancestors (reviewed in Sipiczki, 2000). It is nearly related to *Sch. pombe*, which can show mycelial growth just under very special circumstances or having mutations (Amoah-Buahin *et al.*, 2005; Alonso-Nunez *et al.*, 2005). Now, *Sch. japonicus* can produce both true- and pseudo-mycelium (Sipiczki *et al*., 1998a,b; Bozsik *et al*., 2002; Enczi *et al*., 2007). Furthermore, this species is distantly related to the *Saccharomyces* or *Candida* species, so it can show different and unusual features in its dimorphism and in environmental sensing.

Earlier results suggested that the morphological transition of *Sch. japonicus* required solid substrates (Sipiczki *et al*., 1998b). This feature makes the molecular investigation of hyphae production more difficult. Therefore, aim of this study was to investigate mycelial growth of *Sch. japonicus* and to find those environmental factors which enable the production of long hyphae in liquid media.

Materials and Methods

Strain

Wild-type fission yeast *Sch. japonicus* var. *japonicus* (7-1) (ATCC10660) was obtained from the Czechoslovak Collection of Yeasts, Bratislava, Slovakia (Yukawa and Maki, 1931)(CCY-44-5-1) (CBS 354).

Media

The culture media YEA, YEL (with Scharlau yeast extract 07-079) (pH: 6.7-6.9) and SMA, SML were described previously (Sipiczki and Ferenczy, 1977). YEA* contained 2% glucose instead of 3% glucose. EMMA minimal medium was made according to Mitchison (1970). MB minimal medium was made according to the protocol of Forsburg laboratory (http://www-bcf.usc.edu/~forsburg/media.html). YPL contained 2% glucose, 1% pepton (Scharlau casein trypsic peptone 07-119; tryptone), and 1% yeast extract adjusted to pH 6.7-6.9. YPA contained YPL and 1% agar.

Amino acid containing media, YPL or YPA or EMMA-N source, were supplemented with 10mM amino acid (leucine, or arginine, or isoleucine, or methionine, or proline). FBS containing media (50%): 2 xYPL was diluted with Fetal Bovine Serum solution (1:1), (sterile filtered, Sigma, F7524 Lot. 061M3395). BSA containing media (YPL+1 mg mL^{-1} BSA): BSA solution (NEB B901S, 10mg mL^{-1}) was diluted with the sterile YPL, before inoculation. Extra peptone containing medium (YPL, which contained 1, 2, 5 or 10% peptone) was made with two methods. Ingredients (glucose, yeast extract, water and peptone) were autoclaved together, or glucose, yeast extract and water were autoclaved, which was mixed with filter sterilized peptone solution.

Methods

Growth in liquid media: cultures were grown at 25, 30 or 37^{o}C, without shaking, in 50 ml medium, in 100 ml Erlenmeyer flasks, for one day. Growth on solid media: cultures were grown at 30 or 37^{o}C, for 6-7 days. Heat inactivation of FBS or BSA containing media: the media were heated to boiling point and cooled before inoculation.

Microscopy

Cells were examined and photographed after 24 hours using an Olympus BX40 microscope. Calcofluor was used to stain cell walls and septa (Johnson *et al*., 1979). Vacuolar

morphology was examined by fluorescent vacuolar staining CDCFDA (Yeast Vacuole Marker Sampler Kit, Life Technologies)(Roberts et al. 1991).

Results

Effects of poor condition or osmotic stress on yeast-mycelium transition

Earlier it was demonstrated in solid medium that low concentration (depletion) of nutrition or osmotic stress could lead to morphological switch (Sipiczki*et al.*, 1998b, reviewed in Duran *et al*., 2010). To this end, the effect of minimal media (MB, EMML, SML) which contained just minimal necessities for growth and the osmotic stress (1M sorbitol) were tested. Our results revealed that minimal media did not induce morphological switch at 30^{o}C (Figure 1A), but they enabled production of hyphae at 37^{o}C in small quantities (Figure 1B). Now, the osmotic stress could induce morphological switch effectively both at 30^{o}C and 37^{o}C (Figure 1C,D).

Effect of Fetal Bovine Serum (FBS) on *Sch. japonicus's* dimorphism

As the number of mycelia was not too much in the minimal media and under osmotic stress, we started to test the effect of other supplements. In *C. albicans* Fetal Bovine Serum (FBS) can induce hyphal development (Nantel *et al*., 2002) or germ-tubes (Barlow *et al*., 1974). Therefore, we tested the morphology of *Sch. japonicus* cells in FBS containing medium. We could realise morphological switch after 24h like the *Candida* cells (Figure 2) and this inducing effect was dependent on temperature, since the culture incubated at 37^{o}C resulted in the longest hyphae (Figure 2G-I). Interestingly, detection of FBS is typical only of *Sch.japonicus.* Namely, the closely related *Sch.pombe*' cells showed normal morphology in the presence of FBS (data not show).

To study the concentration dependence, the cells were also cultured in YPL+50% FBS. This higher concentration had very strong effect and it caused "flocculation morphology" in the flask (Figure 3B). Under the microscope we found very long mycelia with strong vacuolisation (Figure 3D,E). Serum-induction in higher concentration had retained its dependence on temperature (data not shown) and it also showed pH dependence, as the hyphal growth was better at lower pH (Figure 3F,G). Active components of FBS can be

peptides (Barlow *et al.*, 1974; Zeng *et al*., 2006), hormones (White and Larsen, 1997) or glucose and a "non-dialysable components" (Hudson *et al*., 2004). If proteins are inducers of the morphological changes, they must be thermosensitive. To test the heat sensitivity of the inducer molecule, we decided to heat the YPL+10% FBS medium to the boiling point before inoculation. In this "boiled" FBS supplemented medium, the yeast cells showed normal morphology (Figure 4B). Thus, the inducing agent of FBS could be eliminated by heating.

Effect of other protein containing ingredients and amino acids on the morphological switch

Assuming that protein(s) of FBS caused the earlier morphological switch, we wanted to test other protein containing ingredients (e.g. BSA or peptone) for the mycelial growth.

In the BSA containing medium, several longer cells and just some very long hyphae were found (Figure 5B), while there were no hyphae and long cells in the control YPL (Figure 5A) or in the boiled YPL+BSA medium at all (data not shown). 2-10% peptone supplemented liquid media gave a similar result (data not shown). Interestingly, this positive effect did not depend on the stage of the peptone (autoclaved or filter sterilized). Furthermore, 10% peptone concentration was rather unfavourable, as several dead cells were found in that culture (Figure 5C). When we tested the effect of 2% peptone in solid medium, it showed stronger effect, as mycelia appeared earlier and were a little bit longer (Figure 5E), compared to the control (Figure 5D).

Peptone may contain not only peptides but also a mixture of amino acids. Furthermore, amino acid-based media are known to induce filamentous growth in *C. albicans* (O'Connor *et al*., 2010) or in *C. parapsilosis* (Kim *et al*., 2006). It was also shown that there are amino acid sensors and transporters, which play an important role in filamentation both in *C. albicans* and in *S. cerevisiae* (Forsberg *et al*., 2001; Brega *et al*., 2004; Bernard and André, 2001). So we investigated the effects of extra amino acids for the morphological switch. Unfortunately, cell morphology was rather normal almost in all cases in liquid (data not shown). Perhaps, a tiny deviation was found in the medium containing arginine, where the cells were more spherical (Figure 5F). In addition, we planned to determine the ability of *Sch. japonicus* cells to use the amino acids as N-source. Therefore, the cells were streaked on the surface of the minimal medium (EMMA) lacking N-source and supplemented with an amino acid. As Figure 5L shows, proline was not a suitable N-source for the fission yeast cells. Now, the cells were able to use arginine, leucine, isoleucine, methionine as N-source (Figure H-K). The size of hyphae was a little bit reduced on the leucine containing medium (Figure

5H), while methionine had rather a positive effect on the length of the hyphae (Figure 5K).

Cell division and mycelium formation at different pH and temperature

Because of the pH and temperature dependence of the FBS induction, we tested their effects under normal growth conditions. The results showed that pH and temperature influenced not only the FBS induced morphological switch, but the normal cell division and uninduced mycelial growth. Namely, lower external pH and higher temperature were favourable to the cell division and production of hyphae (Figure 6,7).

Discussion

The ability of morphological switch is typical of pathogenic fungi (Lo *et al.*, 1997;Stoldt*et al*., 1997; Brand, 2012) and it is generally accepted that hyphal cells are more invasive in several species (Molero*et.al.*, 1998; Whiteway and Oberholzer, 2004; reviewed in Brand, 2012). Thus the study of the genetic background of dimorphism can lead to the understanding of the molecular mechanisms of virulence. As *C. albicans* is diploid and pathogenic, we believe that other model organisms, such as the non-pathogenic and haploid *Sch. japonicus*, can also be very useful in the study of dimorphism. The only difficulty of its molecular investigation was that its mycelial growth seemed to require solid substrates (Sipiczki *et al.*, 1998b). This feature can seriously hamper the study of the molecular background of dimorphism. As dimorphism is in connection with precise environmental sensing, we wanted to find environmental factors which enable the production of long hyphae in liquid media.

The results of this study demonstrate that the cells of fission yeast *Sch.japonicus* can produce hyphae also in liquid medium under special circumstances. The inducer agents are partly similar to the inducers of *C.albicans* and other pathogenic yeasts, since FBS (Figure 2,3) and osmotic stress can induce strong mycelial growth in liquid medium (Figure 1) (Mackenzie, 1962; Kim *et al*., 2000; Pe´rez-Campo and Domıinguez, 2001; O'Connor *et al*., 2010, Duran *et al*., 2010; reviewed in Biswas *et al*., 2007). This suggests that there must be general and common features of the morphological switch even in the distantly related species. Furthermore, the inducer effects of FBS were concentration, temperature and pH pendant (Figure 2,3), similarly to the *C. albicans* cells (Feng *et al*., 1999). Our experiments also revealed that the active component of FBS is thermosensitive (Figure 4B).

As FBS contains BSA and other proteins (Zeng *et al*., 2006; Barlow *et al*., 1974; Hudson *et al*., 2004) which induce mycelial growth in e.g. *C. albicans* and *Y. lipolytica*,(Joshi

et al., 1973ab; Feng *et al.*, 1999; Chen *et al*., 2002; Barlow *et al*., 1974; Szabo, 1999), the effect of BSA and peptone was also tested. These agents enabled the morphological switch, but they did not prove to be as effective as FBS. Namely, 2% peptone was favourable only in solid medium (Figure 5E. Furthermore, 10% peptone concentration in liquid was rather unfavourable (Figure 5C) and BSA also resulted in just few mycelia in the culture. Minimal media (poor conditions) were also ineffective and they caused mycelial growth just when they were combined with 37^{o}C (Figure 1).This suggests that starvation alone is not such an important factor here, in contrast to other species (Gimeno *et al*., 1992; Csank and Haynes, 2000; Amoah-Buahin *et al*., 2005). The study of the effect of amino acids and pH shed light on further differences between *Candida* and *Sch.japonicus*. Namely, Figure 5 showed that metionine had rather a positive effect on hyphae production in solid medium, while it did not affect filamentation of *Candida* (O'Connor *et al*., 2010). Our results also revealed that mycelial growth increased at pH 4-7 (Figure 7), while hyphal formation was inhibited in *Candida* at pH 4 (Konno *et al.*, 2006).These results suggest that there are differences between the two species in the regulation of hyphal growth and pH response of *Sch.japonicus* is rather similar to *C. neoformans* (Wickes *et al*., 1996) or to *U. maydis* (Ruiz-Herrera *et al*., 1995).

In sum, *Sch. japonicus* seems to be a suitable model organism for the further molecular genetic investigation of dimorphism, as its morphological switch can be induced in liquid medium. On the other hand, its study can shed light on both the general and species-specific features of dimorphism.

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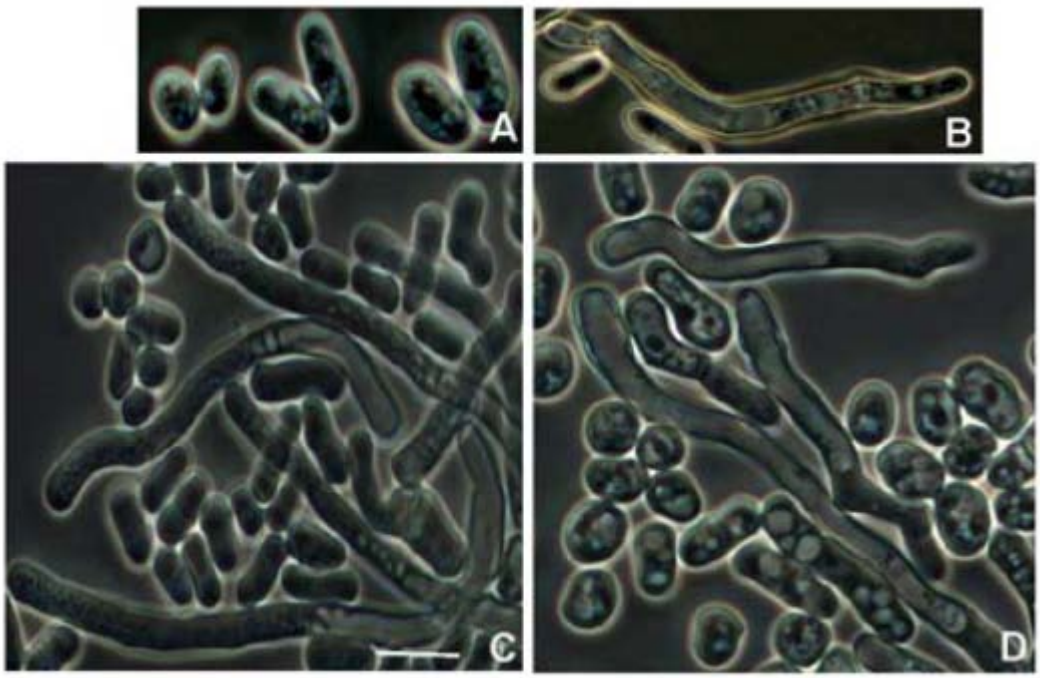

Figure 1. Morphology of the cells in minimal media and in sorbitol containing complete medium. A: minimal medium EMML at 30°C. B: minimal medium EMML at 37°C (Similar cell morphology was found in SML and MB). C:YPL+1M sorbitol at 30°C. D: YPL+1M sorbitol at 37°C. Bar: 10μm

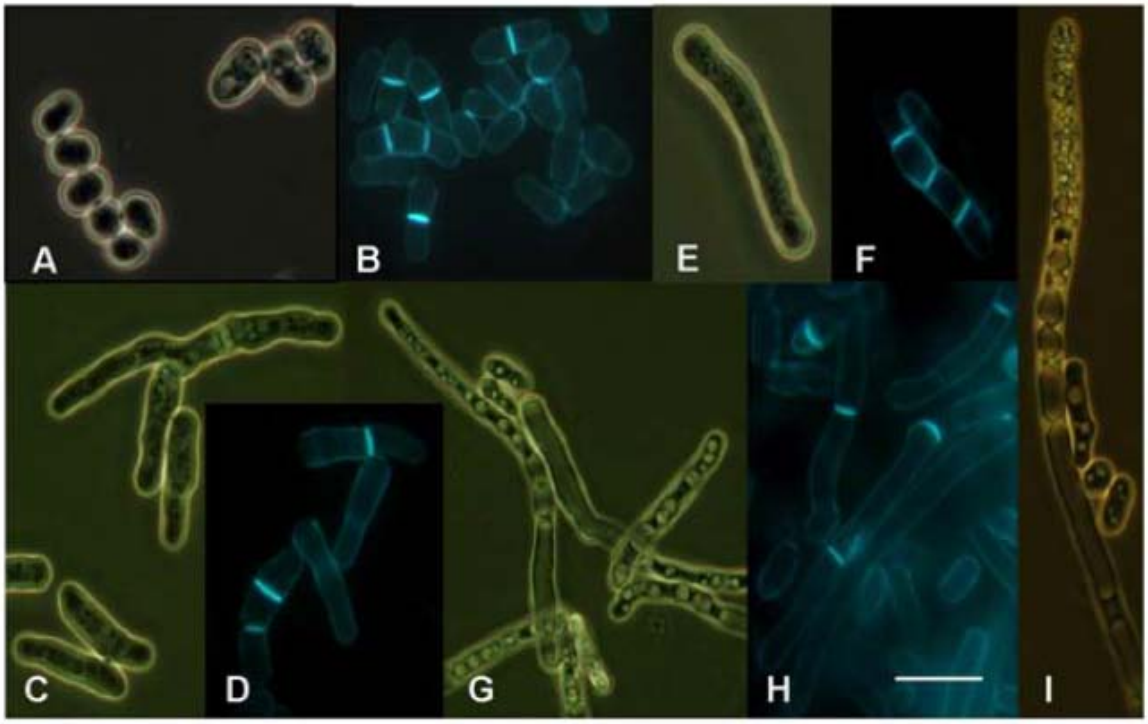

Figure 2. Morphology of the cells in YPL liquid medium containing 10% FBS (without shaking, after 24h). A-B: cells in YPL (control, without serum) at 25-30-37°C. C-D: cells in YPL+FBS at 25°C. E-F: cells in YPL+FBS at 30°C. G-I: cells in YPL+FBS at 37°C. B, D, F and H: calcofluor-stained cells. Bar:10μm.

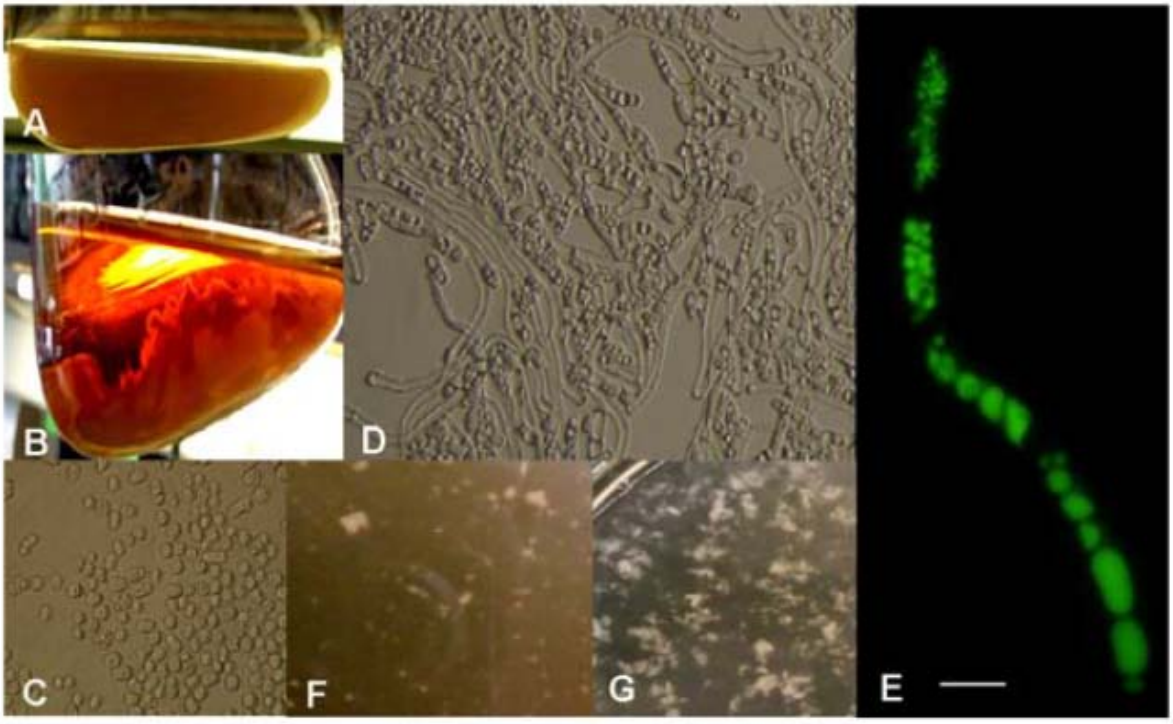

Figure3. *Schizosaccharomyces japonicus* cells cultured in YPL containing 50% FBS (without shaking, at 37°C, overnight culture). A: YPL control. B: “Flocculation” in the flask containing YPL+50% FBS. C: Morphology of the cells in YPL. D-E: Morphology of the cells in YPL+50% FBS. F: Flocculation at pH 6.7. G: „Flocculation” at pH 5.5. C and D Nomarski microscopy. E: Fluorescent vacuolar staining. Bar: 10 μm.

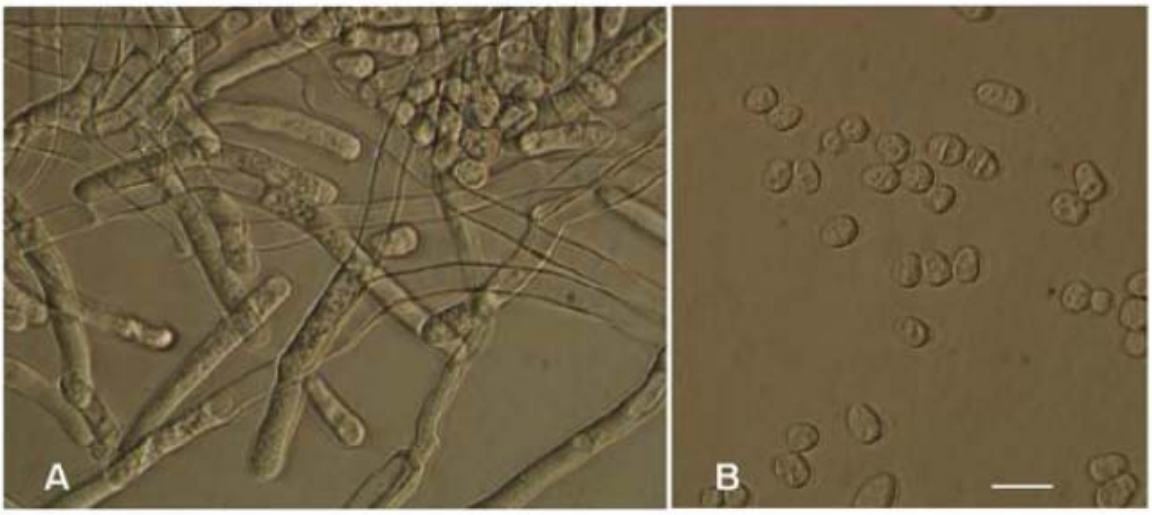

Figure 4. Morphology of the cells in the heated medium (cultured at 37°C, without shaking). A: YPL+10% FBS (control). B: YPL+10% FBS heated to the boiling point. Bar:20 μm.

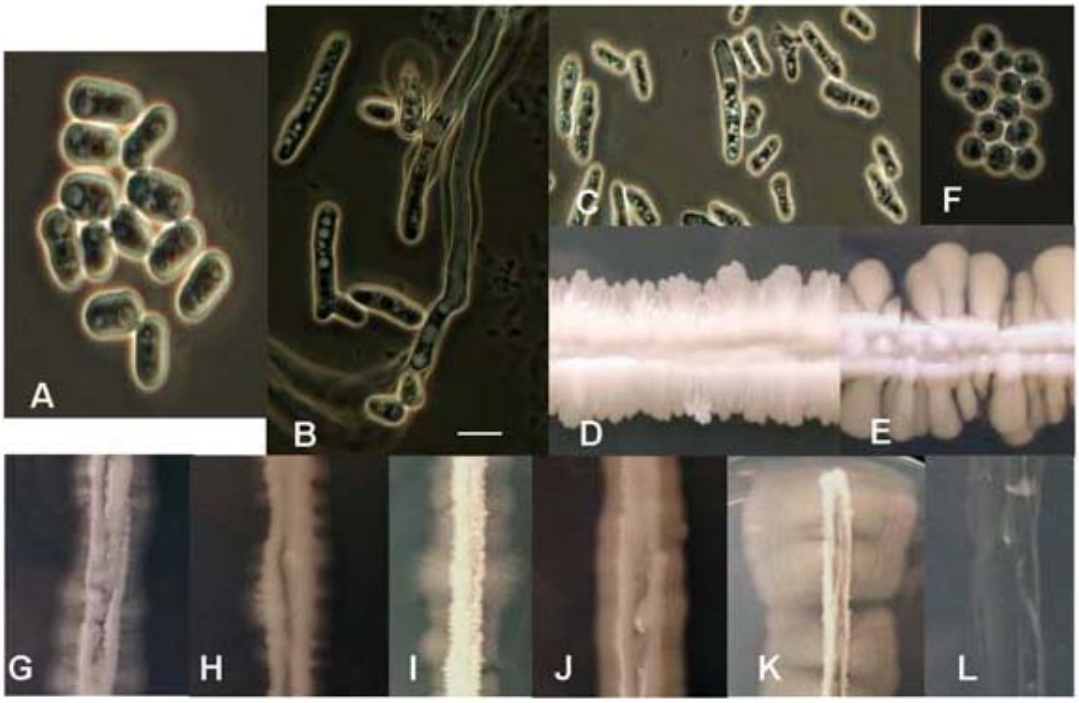

Figure 5. Effect of BSA, peptone and amino acids for cell morphology. A: YPL (control). B: YPL+10% BSA. Bar: 10µm. C: YPL+10% peptone. D: Mycelial growth on solid YEA* (without peptone). E: Mycelial growth on solid YPA (with 2% peptone). F: Cell morphology in YPL+10mM arginine. G: mycelial growth on minimal medium (EMMA). H: mycelial growth on EMMA-N +leucine. I: mycelial growth on EMMA-N +isoleucine. J: mycelial growth on EMMA-N + arginine. K: mycelial growth on EMMA-N + methionine. L: mycelial growth on EMMA-N + proline.

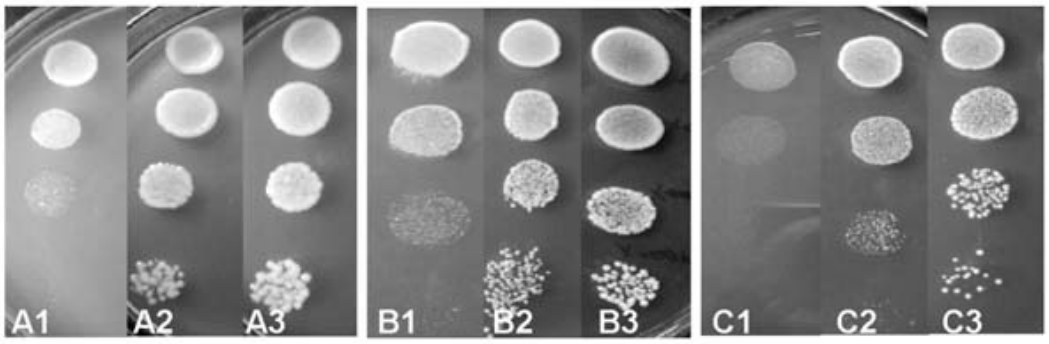

Figure 6. Growth at different temperature and pH. A1:pH4 25°C. A2:pH4 30°C. A3:pH4 37°C. B1:pH7 25°C. B2:pH7 30°C. B3:pH7 37°C. C1:pH8 25°C. C2:pH8 30°C. C3:pH8 37°C.

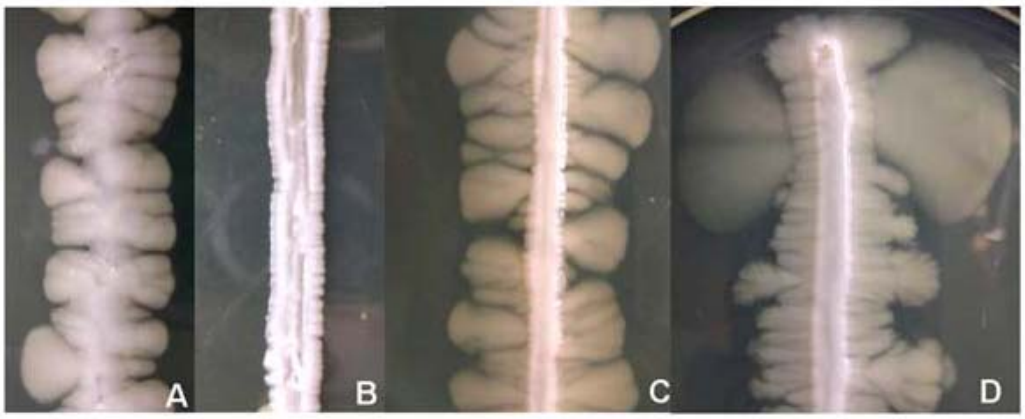

Figure 7. Mycelial growth on YPA at different pH and temperature. A:pH4 37°C. B:pH 8 37°C. C:30°C neutral pH. D:37°C neutral pH.